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DESCRIPTION

IONIZATION SOURCE FOR MASS SPECTROMETRY ANALYSIS

Field of the invention

This invention relates to the field of mass spectrometry, and more particularly to improvements in the chemical ionization source to be applied to mass spectrometers.

Background of the invention

A variety of ionization sources, for the analysis of molecules with medium-high molecular weight (like peptides and proteins) are essential components of modern mass spectrometric instruments. The ionization source transforms neutral molecules into ions which can be analyzed by mass spectrometry.

A mass spectrometer generally has the following components:

- (1) a device, usually a Liquid Chromatograph, for the separation or de-salting of the molecules contained in a sample;
- (2) an ionization source, contained in a chamber, to produce ions from the analyte;
- (3) at least one analyzer or filter which separates the ions according to their mass-to-charge ratio;
- (4) a detector that counts the number of the ions;
- (5) a data processing system that calculates and

plots a mass spectrum of the analyte.

The mass spectrometry techniques currently used for the analysis of macromolecules and, especially, proteins and peptides are based on the Electrospray Ionization (ESI) (U.S. Patent No 5756994; Cunsolo V, Foti S, La Rosa C, Saletti R, Canters GW, Verbeet M. Ph. *Rapid Commun. Mass Spectrom.* 2001; 15: 1817; Wall DB, Kachman MT, Gong SS, Parus SJ, Long MW, Lubman DM. *Rapid Commun. Mass Spectrom.* 2001; 15: 1649; Fierens C, Stöckl D, Thienpont LM, De Leenheer AP. *Rapid Commun. Mass Spectrom.* 2001; 15: 1433; Li W, Hendrickson CL, Emmett MR, Marshall AG. *Anal. Chem.* 1999; 71: 4397; Fierens C, Stöckl D, Thienpont LM, De Leenheer AP. *Rapid Commun. Mass Spectrom.* 2001; 15: 451) and Matrix Assisted Laser Desorption Ionization (MALDI) (U.S. Patent No 5965884; Cozzolino R, Giorni S, Fisichella S, Garozzo D, La fiandra D, Palermo A. *Rapid Commun. Mass Spectrom.* 2001; 15: 1129; Madonna AJ, Basile F, Furlong Ed, Voorhees KJ. *Rapid Commun. Mass Spectrom.* 2001; 15: 1068; Basile A, Ferranti P, Pocsfalvi G, Mamone G, Miraglia N, Caira S, Ambrosi L, Soleo L, Cannolo N, Malorni A. *Rapid Commun. Mass Spectrom.* 2001; 15: 527; Galvani M, Hamdan M, Rigetti PG. *Rapid Commun. Mass Spectrom.* 2001; 15: 258; Ogorzalek Loo RR, Cavalcali JD, VanBogelen RA, Mitchell C, Loo JA, Moldover B, Andrews PC. *Anal. Chem.* 2001; 73:

4063).

Both techniques are highly effective for the production of ions of biomolecules in the gas phase, to be subsequently analyzed by Mass Spectrometry (MS).

5 In the case of ESI, multicharge ions of medium/high molecular weight compounds are produced. The mass of macromolecule compounds is then obtained using specific software algorithms.

Mass spectrometry represents an essential  
10 technology in the analytical field. It is usually coupled with other separative techniques, so as to identify chemical compounds and quantify complex biological mixtures. Proteins, for instance, are first separated, collected and then digested with Trypsin. The  
15 masses of the resulting peptides are determined by mass spectrometry (normal scan MS or tandem mass spectrometry MS/MS). In the case of the MS/MS approach, peptide ions of a single  $m/z$  ratio are fragmented by collision induced dissociation (CID) and then analyzed using  
20 various mass analyzers (triple quadrupole, ion trap, Fourier transform-ion cyclotron resonance). Each peptide gives origin to specific mass patterns for a given amino acid sequence. The peptide sequences can be obtained by computer analysis of the data using a dedicated software  
25 (database search and de novo sequence software). In

order to obtain good MS/MS spectra doubly charge peptide ions are preferably fragmented (Cramer R, Corless S. *Rapid Commun. Mass Spectrom.* 2001; 15: 2058). The electrospray and MALDI techniques when are applied to the analysis of peptides with high molecular weight (2000-4000 Thompson (Th)) using the MS/MS approach have some limitations. For instance, when proteins or peptides with high molecular weight are analyzed, ESI multicharge ions are produced. These ions give rise to complex fragmentation spectra, difficult to interpret. For this reason only peptides with a maximum of 15 amino acidic residues can be analyzed by tandem mass spectrometry. In the case of MALDI only mono-charge ions are usually obtained. If the MALDI source is coupled with Time of Flight Mass Analyzer (TOF) the technique used to fragment the ions is the post source decay (PSD). This fragmentation technique give rise to some additional problems; in order to obtain good fragmentation spectra it is usually necessary to use peptide derivatization. A MALDI atmospheric pressure source has recently been coupled with an ion trap analyzer. This configuration makes possible the structural analysis of peptides by MS/MS and MS<sup>3</sup>. However, it must be emphasized that the MALDI source produces, mainly, mono-charge peptide ions that produce

fragmentation spectra more complex and less specific than those obtained by fragmentation of the bi-charge ions.

Another problem that affect both MALDI and ESI techniques is represented by the decrease in sensitivity when salts are present in the sample. In the case of ESI the problem may be solved by coupling the mass spectrometer with a pre-analytical separation step, such as by the use of an High Performance Liquid Chromatographer (HPLC) or other de-salting techniques. This obviously introduces another step in the whole procedure of analysis. The HPLC technique on the other hand cannot be used for the case of MALDI because in this case it is necessary to co-crystallize the analyte with a matrix molecule. Salts contained in the sample must, however, be eliminated before of the crystallization step by well known additional treatments of the sample.

Purpose and description of this invention and improvements over the prior art

The present invention is based on the introduction of a device for the ionization of neutral molecules in the gas phase. The device comprises an active surface carrying element that, according to this invention, is inserted in the ionization chamber. This technique has

been named by us "Surface Activated Chemical Ionization" (SACI). SACI technique allows the ionization to be performed at atmospheric pressure.

Use of an atmospheric-pressure ionization has already been proposed and is known as the APCI technique. APCI instrument makes use of a needle-shaped corona discharge electrode inserted inside the ionization chamber. However, the high energy of the corona discharge electrode leads to the macromolecules fragmentation. The main problem of this method is the lower sensitivity with respect to ESI and MALDI techniques.

We have now surprisingly found that introducing into the ionization chamber a plate-like active-surface carrying element can bring to unexpected results in term of high sensitivity and possibility to detect molecules having a molecular weight in a broad range of values.

According to the invention, the solution containing the analyte is injected in the SACI source through an inlet aperture. The sample is nebulized by a gas flow and vaporized by heating. The ionization chamber contains an active surface carrying element onto which the vaporized molecules of the analyte bump, so that the analyte becomes ionized. This active surface can be made of various materials (steel, glass, quartz etc),

both electrically conductive or not. Different molecules can also be bound or absorbed over the surface to improve the ionization process ( $H_2$ ,  $D_2O$  and various acid and basic molecules). The analyte neutral molecules which are present in gas phase are ionized by various physical-chemical interactions which take place on the surface. Surface properties and function in catalyzing various kind of reactions is well known (U.S. Patent No 5503804; U.S. Patent No 5525308; U.S. Patent No 5856263; U.S. Patent No 5980843).

An interesting use of a surface in mass spectrometry is the Surface Enhanced Laser Desorption Ionization (SELDI) (U.S. Patent No 6020208; U.S. Patent No 6124137; U.S. Patent No 20020060290; U.S. Patent No 5719060). In this case the probe of MALDI mass spectrometer carries an immobilized affinity reagent which binds the analyte on its surface. Furthermore an energy absorbing material is added to the dried sample and Laser Desorption Ionization mass spectrometry is used to analyze the sample. This technique however differs from the SACI because of the fact that the sample can be prepared in advance by deposition over the surface, so that this analysis is quite time consuming. Some ionization source make use of an electrical potential applied to a needle to ionize the sample, in

gas phase, by using the corona discharge effect (U.S. Patent No 6407382; U.S. Patent No 5684300; U.S. Patent No 6294779; U.S. Patent No 5750988; U.S. Patent No 6225623; U.S. Patent No 5756994; U.S. Patent No 20020074491; U.S. Patent No 20020048818; U.S. Patent No 20020011560; U.S. Patent No 4849628) ..

The use of the SACI ionization source which is disclosed in this invention, represents a key improvement for the production of ions with high molecular weight and low charge (bi-charge ions are usually much abundant). The innovative aspect of this invention over the previous known art can be so summarized:

15 a) Analytes with higher molecular mass can be studied since the technique is able to generate ions with high molecular weight and low charge, an essential feature useful for obtaining the mass of macromolecule compounds. Best results can be obtained if the source is  
20 coupled with a mass analyzer with high mass range like Fourier Transform - Ion Cyclotron Resonance (FT-ICR) or Time Of Flight (TOF).

b) A higher sensitivity can be obtained in the analysis of molecules with high mass and low charge  
25 (typically bi-charge ions). This is particularly useful



for analyzing biological compounds, like proteins and peptides, which are frequently present at low concentration in biological samples (tissues, urine, etc).

5       c) The new technique makes it now possible to analyze molecules with medium/high mass and low charge (typically the bi-charge ions), by the MS/MS approach. This feature is useful to characterize proteins and high molecular weight peptides. In fact we have shown that  
10 peptides containing more than 15 amino acidic residues can be studied. This is particularly useful for the characterization of peptides with high mass, originated by missed cleavage during the enzymatic digestion reaction.

15       d) The SACI ionization source is much less affected by the presence of salts than the ESI and MALDI sources. The new invention makes it now possible to analyze liquid biological samples, which usually contain salts or buffers, by direct infusion into the mass  
20 spectrometer without using an HPLC systems or other desalting procedures. This is particularly useful for analyzing samples in high throughput applications. Samples containing a high concentration of salts are well known to give rise to serious problem when the ESI  
25 or MALDI techniques are used.

Table 1 summarize the critical improvements obtained by the application of SACI vs ESI technique.

Table1: A summary of the critical improvements  
5 obtained by the application of SACI vs ESI techniques

SACI vs ESI	
- Detect ions with high mass and low charge	- Detect multicharge ions with high mass
- High throughput	- Pre-analytical steps limit throughput
- "Tolerant" of salts	- Less tolerant of salts
- Can sequence peptides with high molecular weight (more than 15 amino acid)	- Can not sequence peptides longer than 15 amino acid
- High sensitivity, Low chemical noise	- Higher chemical noise
	- Lower sensitivity

#### Brief description of the drawings

Figure 1: A schematic representation of the new  
10 device, i.e. the Surface Activated Chemical Ionization source (SACI).

#### Figure 2:

a) Mass spectrum, obtained by direct infusion in the mass spectrometer using the SACI technique, of a

sample containing a mixture of five peptides (peptide YY fragments 13-36 obtained from Sigma catalog number P6613, MW 3014 Da; Diabetes associated peptide fragment 8-37 obtained from Sigma catalog number. D6170, MW 3200 Da; Gastrin releasing peptide human obtained from Sigma catalog number G8022, MW 2859 Da; Phospholipase 2 activating peptide obtained from Sigma catalog number G1153, MW 2330 Da; and Vasoactive Intestinal Peptide Fragment 6-28 obtained from Sigma catalog number V4508, MW 2816 Da) acquired in the 400 - 4000 Th range. The solution concentration of each peptide was  $10^{-7}$  M. The counts/s value was  $10^6$  and the S/N ratio of the most abundant peak was 500. No salts were added in the pure  $H_2O$  solution containing the peptides.

b) Mass spectrum, obtained by direct infusion in the mass spectrometer using the ESI technique, of the same solution as in (a),. The counts/s value was  $10^5$  and the S/N ratio of the most abundant peak was 100. A much higher chemical noise can be observed in this case, leading to a decrease of the S/N ratio. Using the SACI ionization source the mono and bi-charge ions were mainly obtained, whereas using the ESI ionization source only the tri-charge ions can be detected. It must be emphasized that the multicharge phenomenon, which takes place by using the ESI source, leads to a compression of

the mass signals. An overlap of the multicharge signals, which usually takes place for molecules with high molecular weight is also observed.

Figure 3:

5 a) Mass spectrum, obtained by direct infusion in the mass spectrometer using the SACI technique, of a standard protein (Cytochrome C) acquired in the 4000 - 14000 Th range. The protein was obtained by Sigma-Aldrich (catalog number 10,520-1) and diluted in H<sub>2</sub>O so  
10 to obtain a concentration of 10<sup>-7</sup> M. The counts/s value was 10<sup>6</sup> and the S/N ratio of the most abundant peak was 300.

b) Mass spectrum obtained by direct infusion in the mass spectrometer using the ESI technique, of the same  
15 solution as in (a). No signals were detected in this case. This is due to the extensive multicharge phenomenon that takes place in the ESI ionization source.

c) Multicharge distribution of the Cytochrome C  
20 protein obtained using the ESI ionization source. The multicharge distribution is usually compressed in the first region of the spectrum (100-2000 Th) thus leading to a decrease of the sensitivity.

Figure 4:

25 a) Tandem mass spectrum, obtained by using the

SACI technique, of the bi-charge ion of Vasoactive Intestinal Peptide Fragment 6-28 at  $m/z$  1409.

b) Tandem mass spectrum of the same solution, obtained using the ESI technique. The tri-charge ion at  $m/z$  940 was fragmented. In the case of the fragmentation of the tri-charge ion few fragmentation peaks were obtained.

Figure 5:

a) Mass spectrum, obtained by direct infusion in the mass spectrometer using the SACI technique, of a sample containing a mixture of five peptides, as in figure 2a, acquired in the 400 - 4000 Th range. The solution had a ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) concentration of 50 mmol/L. The counts/s value was  $10^6$  and the S/N ratio of the most abundant peak was 500.

b) Mass spectrum obtained by direct infusion in the mass spectrometer using the ESI technique, of the same solution as in (a). The counts/s value was  $10^5$  and the S/N ratio of the most abundant peak was 100. In the case of the ESI technique a high chemical noise leads to decrease the quality of the spectrum. The multicharge phenomenon also takes place leading to decrease the quality of the spectrum.

Figure 6:

a) Mass spectrum, obtained by direct infusion in

the mass spectrometer using the SACI technique, of a peptide mixture obtained by tryptic enzymatic digestion of Cytochrome C, in the presence of 50 mmol/L  $\text{NH}_4\text{HCO}_3$ . The identified peptides are marked by their amino acidic intervals as compared with the original protein sequence. The initial (before tryptic digestion) concentration of the protein was  $10^{-7}$  M. The counts/s value was  $10^6$  and the S/N ratio of the most abundant peak was 450.

10        b) Mass spectrum, obtained by direct infusion in the mass spectrometer using the ESI technique, of the same solution. The counts/s value was  $10^5$  and the S/N ratio of the most abundant peak was 100. In this case a higher chemical noise as compared with (a) is present.

15        Moreover, in the case of the ESI ionization source spectrum, less peptide signals were detected.

Figure 7:

a) Mass spectrum, obtained by direct infusion in the mass spectrometer using the SACI technique and in  
20        absence of salts, of a sample containing a mixture of five peptides as in Figure 2a. The counts/s value was  $10^6$  and the S/N ratio of the most abundant peak was 500.

b) Mass spectrum obtained by direct infusion in the mass spectrometer using the SACI technique, of a sample  
25        containing a mixture of five peptides as in (a), but

containing 50 mmol/L  $\text{NH}_4\text{HCO}_3$ . It must be emphasized that this buffer is commonly used for biological application (for example to perform the tryptic digestion). The counts/s value was  $10^6$  and the S/N ratio of the most abundant peak was 500. It should be noted that the presence of the buffer does not lead to a decrease in the quality of the spectrum or a higher chemical noise.

Figure 8:

a) Mass spectrum, obtained by direct infusion in the mass spectrometer using the ESI technique, of a sample containing a mixture of five peptides as in figure 2b. The counts/s value was  $10^5$  and the S/N ratio of the most abundant peak was 100.

b) Mass spectrum, obtained by direct infusion in the mass spectrometer using the ESI technique, of the same sample as in (a) but in the presence of 50 mmol/L  $\text{NH}_4\text{HCO}_3$ . The counts/s value was  $10^5$  and the S/N ratio of the most abundant peak was 100. It can be seen that the presence of the buffer leads a decrease of the peaks at m/z 778, 954, 1006 and 1068.

**Description of a preferred embodiment of the present invention and application examples**

The SACI source described in this invention and schematically represented in Figure 1 produces ions that can be analyzed in a mass spectrometer. The spectrometer

comprises the ionization source, the analyzer or filter for separating the ions by their mass-to-charge ratio, a detector for counting the ions and a data processing system. Since the structure of the spectrometer is conventional, it will not be described in more detail, but the ionization source device which is the subject of the present invention. The ionization source of the invention, on its turn, does not substantially differ, in its structure, from the known devices of this kind, so that a schematic representation thereof will be sufficient for the skilled man in this art to understand how it is constructed and works.

The ionization source device of the invention comprises an inlet assembly 11 which is in fluid communication with an ionization chamber 3.

The ionization chamber 3 comprises an outlet orifice, generally less than 1 mm in diameter, for communicating between the ionization chamber and the analyzer or filter. Generally, the angle between the axis of the inlet assembly 11 and the axis passing through said orifice is about  $90^\circ$ , but different relative positions can also be envisaged. Inside the ionization chamber 3 is positioned a plate 4. The plate 4 has at least one active surface 4' which faces the internal aperture of the inlet assembly 11. Preferably,



the plate 4 is inclined of an angle which allows the analyte to be reflected, once ionized, towards the outlet orifice bringing to the analyzer or filter, so that the highest number of ions can reach the analyzer (mirror effect). This will strongly improve the sensitivity of the method. The said inclination angle will depend of course on the relative position of the axes of both inlet assembly 11 and outlet orifice. For example, if such axes form an angle of  $90^\circ$ , the element 4 will be  $45^\circ$  inclined.

The plate 4 can have different geometries and shapes, such as squared, rectangular, hexagonal shape and so on, without departing for this from the scope of the present invention. It has been found that the sensitivity of the analysis increases when the active surface 4' is increased. For this reason, the plate 4 surface will range preferably between 1 and 4  $\text{cm}^2$  and will be generally dictated, as the highest threshold, by the actual dimensions of the ionization chamber 3. While maintaining the dimension of the plate 4 fixed, the active surface 4' area can be increased in various ways, for example by creating corrugations on the surface 4'. In particular cases, such as the case wherein low molecular weight molecules must be analyzed, high electrical field amplitude is required. In such cases,

it may be advantageous to provide the active surface 4' with a plurality of point-shaped corrugations, in order to increase in such points the electrical field amplitude.

5       The plate 4 has generally a thickness of between 0.05 and 1 mm, preferably of between 0.1 and 0.5 mm.

      The active surface 4' can be made of various materials, either of electrically conductive or non-conductive nature. Preferred materials can be a metal  
10   such as iron, steel, copper, gold or platinum, a silica or silicate material such as glass or quartz, a polymeric material such as PTFE (Teflon), and so on. When the active surface 4' is comprised of a non-conductive material, the body of the plate 4 will be  
15   made of an electrically conductive material such as a metal, while at least a face thereof will be coated with the non-conductive material in form of a layer or film to create the active surface 4'. For example, a stainless steel plate 4 can be coated with a film of  
20   PTFE. It is in fact important that, even if of non-conductive nature, the active surface 4' be subjected to a charge polarization. This will be achieved by applying an electric potential difference to the body plate, thus causing a polarization to be created by induction on the  
25   active surface 4' too. On the other hand, if the surface

4' is of electrical conductive nature, the plate 4 does not need to be coated. In this case, a good performance of the ionization source of the invention can be achieved even without applying a potential difference, 5 i.e. by maintaining the surface 4' at ground potential and allowing it to float.

The plate 4 is linked, through connecting means 5, to a handling means 6 that allows the movement of the plate 4 in all directions. The handling means 6 can be 10 moved into the ionization chamber and also can be rotated. The connecting means 5 can be made of different electrically conductive materials and can take various geometries, shapes and dimensions. Preferably, it will be shaped and sized so as to facilitate the orientation 15 of the plate 4 in an inclined position. In this case, the connecting means 5 will have a step-like shape (as shown in figure 1). The plate 4 is electrically connected to power supply means 20 in order to apply a potential difference to the active surface 4'.

20 Coming now to the description of the inlet assembly 11, the liquid sample containing the analyte is introduced into the chamber through the sample inlet hole 10. The inlet assembly 11 comprises an internal duct, open outwardly via the said inlet hole 10, which 25 brings to a nebulization region 12. The said

nebulization region is in fluid communication with at least one, typically two gas lines 14, 15 (typically, the gas is nitrogen) which intercepts the main flow of the sample with different angles, so that to perform the functions of both nebulizing the analyte solution (angle >45°) and carrying it towards the ionization chamber 3 (angle <45°). Downstream to the said nebulization region 12, a heating region 13 is provided. The heating region 13 comprises heating means, such as a heating element connected to a power supply connector 16. The vaporized analyte is thus heated at temperatures ranging from 200°C and 450°C, preferably of between 250°C and 350°C. The internal duct of the inlet assembly 11 ends into the ionization chamber 3 in a position which allows the vaporized and heated analyte to impact the active surface 4' of the plate 4, where the ionization of the neutral molecules of the analyte takes place. Without being bound to any particular theory, it is likely that a number of chemical reactions take place on the surface: proton transfer reactions, reaction with thermal electron, reaction with reactive molecules located on the surface, gas phase ion molecule reactions, molecules excitation by electrostatic induction. It is also possible that the dipolar solvent is attracted from the active surface 4' by means of the

charge polarization induced on it and so provide a source of protons that react with the analyte molecules to form ions. As said before, the plate 4 can be allowed to float - only if the active surface 4' is electrically  
5 conductive, since in this case an electron exchange flow can be established between the solvent and the surface 4' - or a potential difference can be applied. Such a potential difference, as absolute value, will preferably be in the range of from 0 and 1000 V (in practice, can  
10 range between -1000 V and + 1000 V, depending on the kind of polarization that is required on the active surface 4'), preferably of from 0 and 500 V, more preferably of from 0 and 200 V. High voltage, such as about 200 V, allows the ionization yield to be  
15 increased. The possibility given by the present invention device to work both with and without a voltage to be applied to the analyte is of pivotal importance. In fact, in some instances, there are molecules that do not suffer a strong electrical field, such as the  
20 macromolecules or even some small molecules like amphetamines, which degrade in such strong conditions. In general, it can be said that the absence of a voltage applied to the plate 4 avoids redox reactions to the analyte.

25 For the reasons seen above, it is important that

the solvent in which the analyte is dissolved be a dipolar solvent having acidic protons. Preferred solvents are H<sub>2</sub>O, alcohols such methanol or ethanol, acetonitrile.

5        The impact angle of the analyte onto the active surface 4' will be preferably 45° or less. Low impact angle values allow a better contact between the analyte and the active surface, thus improving the ionization performance.

10       In a preferred embodiment of the invention, the analyte solution also contains aminoacids such as glycine, lysine, istidine, aspartic acid and glutammic acid, which have the function of proton donors to promote the analyte ionization.

15       The ions so formed are reflected and directed to the analyzer 1 through the outlet orifice, as described above.

20       The essential feature of the invention consists in the introduction of a n active surface 4' in the vaporization chamber 3, that enhances the ionization of the neutral analyte molecules present in gas phase. The SACI can be considered a soft ionization source, which can be of particular interest in several applications, such as in the field of drugs and anti-doping analysis.

25       It should be understood that the above description

is intended to illustrate the principles of this invention and is not intended to limit any further modifications, which can be made following the disclosure of this patent application by people expert  
5 in the art.

The following, not limiting, examples are described to illustrate the novelty and usefulness of the invention.

EXAMPLE 1: The observation of ions in the High Mass  
10 Range

A  $10^{-7}$  M solution of Cytochrome C protein (MW: 12361) has been analyzed by direct infusion. Figure 3a shows the protein signals obtained using the new SACI ionization source. The mono-charge, bi-charge and tri-  
15 charge ions were clearly detected using positive acquisition mode. This compares with results on the same solution achieved by the use of the ESI ionization source (Figure 3b). In this latter case no multicharge distribution was detected in the 4000-14000 Th range. In  
20 fact signals obtained in this region of the spectrum by the use of the ESI ionization source are due to the chemical noise of the solvent. It is well known that the ESI ionization source cannot be used to analyze molecules with high molecular weight and low charge.  
25 Thus the ESI technique has serious limits for analyzing

biological molecules with high molecular weight (like proteins). In order to overcome this limitation the MALDI ionization source is used since. The ionization source of MALDI is able to produce low charge ions in the range 1000 - 300000 Th. The application of MALDI technique, however, requires co-crystallization of the analyte with a matrix molecule. To ionize the sample a laser light that is mainly adsorbed by the matrix molecule is ordinary used. A micro explosion process (ablation) take place on the surface of the crystal and the excited matrix molecules ionize the sample molecules in gas phase (soft ionization reaction). For this reason a HPLC or similar on line separation methods cannot be used in the MALDI approach. It must be emphasized that the SACI ionization source is able, like the MALDI source, to generate ions with high molecular weight and low charge, but, in addition, it can be coupled in line with HPLC or other separatory methods.

EXAMPLE 2: An application of SACI technique to the analysis of high molecular weight peptides

Five high molecular weight standard peptides with molecular mass in the 2000 - 4000 Da range were analyzed. The results obtained using the SACI source are shown in Figure 2a. As can be seen the mono and bi-charge peptide ions were clearly detected. The peptides



were analyzed also by a mass spectrometer using the ESI ionization source (Figure 2b). In this case the tri-charge peptide ions are the most abundant species. These species are located in a region of the spectrum (500-  
5 1100 Th) in which the chemical noise is high leading to decrease the S/N ratio.

The mass analyzer used to perform both experiments was an ion trap (LCQ<sup>XP</sup>, ThermoFinnigan, USA) able to detect the signals in the 100-4000 Th and 1000-20000 Th  
10 range. The mass acquisition range can also be extended by coupling the SACI ion source with other kind of mass analyzer (for example TOF or FT-ICR) provided with a high mass acquisition range.

EXAMPLE 3: Increase in sensitivity provided by the  
15 new ionization source

The SACI ionization source first described in the present invention is characterized by a higher sensitivity, as compared to the ESI technique, in the analysis of liquid samples of proteins and peptides.  
20 Figure 2a and 3a show the spectra obtained by direct infusion of solutions of five high molecular weight peptides (Figure 2a) and Cytochrome C (Figure 3a). A LCQ<sup>XP</sup> (ThermoFinnigan, USA) provided with SACI ionization source was used. The solution concentration  
25 of each standard peptide and of the Cytochrome C was 10<sup>-</sup>

<sup>7</sup> M and the counts/s value was  $10^6$  with a S/N ratio of the most abundant peak of 500 for the high molecular weight peptides and 300 for the Cytochrome C protein. The comparison of these results with those obtained, for the same solutions, using the ESI ionization source (Figure 2b and 3b) shows that the SACI ionization source increases the sensitivity. As can be seen for the case of the ESI spectra of the same high molecular weight peptides (Figure 2b) the most abundant signals (tri-charge ions) are detected in the 500-1100 Th range, due to the multicharge phenomenon. Furthermore, the chemical noise is higher (S/N ratio of the most abundant peak = 100) using the ESI technique than that obtained by the SACI ionization source (S/N ratio of the most abundant peak = 500).

In the spectrum of the Cytochrome C, obtained by the ESI ionization source (Figure 3b), no protein signal has been detected in the 4000-14000 Th range. This is due to the extensive multicharge phenomenon that takes place in the ESI ionization source. For this reason the multicharge distribution is usually compressed in the 100-2000 Th range (Figure 3c) where the chemical noise is higher.

EXAMPLE 4: Characterization of high molecular weight peptides

The tandem mass spectrometry (MS/MS) of bi-charge ions, that are abundantly produced by the SACI source, can be further characterized. In Figure 4a the SACI-MS/MS spectrum of the bi-charge ion of Vasoactive Intestinal Peptide Fragment 6-28 is shown. The bi-charge ion was isolated into the ion trap analyzer and fragmented by Collision Induced Dissociation (CID). The results of the peptide identification and its relative statistical correlation score, by the use of the SEQUEST database search program, were as follows:

Peptide	Xcorr	DeltCn
Vasoactive Intestinal Peptide Fragment 6-28	3.5382	0.204

Xcorr is a spectra correlation score and DeltCn is the 1.0 - normalized correlation score. A correctly identified peptide has a value of Xcorr score higher than 3. The peptide was also analyzed using the ESI ionization source (Figure 4b). In this case the bi-charge peak at m/z 1409 had a too weak intensity to obtain an MS/MS spectrum. Thus, the tri-charge ion at m/z 940 was fragmented. The statistical correlation score and the DeltCn in this case were as follows:

Peptide	Xcorr	DeltCn
Vasoactive Intestinal Peptide Fragment 6-28	1.2280	0.608

As can be seen by the Xcorr and DeltCn scores so calculated, the peptide characterization is statistically more accurate using the SACI-MS/MS spectrum obtained fragmenting the bi-charge ions at m/z 1409.

#### EXAMPLE 5: Effect of salts on sensitivity

Figure 5a and 6a show the mass spectra of a solution of five standard peptides and of peptides obtained by Cytochrome C tryptic digestion all in 50 mmol/L  $\text{NH}_4\text{HCO}_3$  buffer. The SACI ionization source was used. In both cases the solution concentration was  $10^{-7}$  M. The counts/s value was  $10^6$  and the S/N ratio was 500 in the case of the high molecular weight peptides and 450 in the case of Cytochrome C peptides. The results obtained using the ESI ionization source is shown in Figure 5b and 6b. As can be seen in these latter cases the mass spectra show a high chemical noise, due to the presence of the buffer. This leads to a decrease in sensitivity as compared to that obtained by the use of SACI ionization source. In fact the counts/s value was an order of magnitude lower ( $10^5$ ) and the S/N ratio of

the most abundant peak (100) is 5 times lower.

In order to show that the S/N ratio is not affected by salts, Figure 7 reports the mass spectra of five high molecular weight peptides acquired without (Figure 7a) and with (Figure 7b) salts in the sample solutions. The SACI ionization source was used in both cases. As can be seen salts do not lead to a decrease of the spectrum quality. This fact is very important when biological mixtures are analyzed. In fact these mixtures almost always contain salts or buffers (as for example  $\text{NH}_4\text{HCO}_3$  used for the tryptic digestion) that give rise to well known effect on the ESI mass spectra.

Figure 8 shows the spectra obtained by analyzing the high molecular weight peptide solutions in absence (Figure 8a) and in presence (Figure 8b) of salts by the standard ESI technique. In both cases the spectra show a higher chemical noise than in those obtained using the SACI ionization source (respectively shown in Figure 7a and 7b). The addition of the  $\text{NH}_4\text{HCO}_3$  buffer to the solution analyzed by the ESI technique decrease the peptide signals at  $m/z$  1068, 1006, 778 and 954. For this very reason an HPLC or other separation steps system is coupled with the ESI ionization source. A chromatographic analysis, however, takes time and increases the number of manipulation of the sample

before analysis. This is a limit especially when many samples must be analyzed.